JOURNAL OF VIROLOGY, July 2010, p. 6615–6625 Vol. 84, No. 13
0022-538X/10/$12.00 doi:10.1128/JVI.00417-10

Regulation of Hepatitis C Virus Translation and Infectious Virus Production by the MicroRNA miR-122[▽]

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Received 25 February 2010/Accepted 16 April 2010

miR-122 is a liver-specific microRNA that positively regulates hepatitis C virus (HCV) RNA abundance and is essential for production of infectious HCV. Using a genetic approach, we show that its ability to enhance yields of infectious virus is dependent upon two miR-122-binding sites near the 5′ end of the HCV genome, S1 and S2. Viral RNA with base substitutions in both S1 and S2 failed to produce infectious virus in transfected cells, while virus production was rescued to near-wild-type levels in cells supplemented with a complementary miR-122 mutant. A comparison of mutants with substitutions in only one site revealed S1 to be dominant, as an S2 but not S1 mutant produced high virus yields in cells supplemented with wild-type miR-122. Translation of HCV RNA was reduced over 50% by mutations in either S1 or S2 and was partially rescued by transfection of the complementary miR-122 mutant. Unlike the case for virus replication, however, both sites function equally in regulating translation. We conclude that miR-122 promotes replication by binding directly to both sites in the genomic RNA and, at least in part, by stimulating internal ribosome entry site (IRES)-mediated translation. However, a comparison of the replication capacities of the double-binding-site mutant and an IRES mutant with a quantitatively equivalent defect in translation suggests that the decrement in translation associated with loss of miR-122 binding is insufficient to explain the profound defect in virus production by the double mutant. miR-122 is thus likely to act at an additional step in the virus life cycle.

Hepatitis C virus (HCV) is a hepatotropic human virus classified within the family *Flaviviridae* and possessing a positive-sense, single-stranded RNA genome of about 9.6 kb that encodes a large polyprotein of approximately 3,010 amino acids. It is an important human pathogen, with 1 in 50 persons worldwide being chronically infected with HCV and at risk for developing cirrhosis and hepatocellular carcinoma (22). The polyprotein is co- and posttranslationally processed by cellular and viral proteases into at least 10 individual proteins, including the nucleocapsid core protein; two glycoproteins, E1 and E2; a putative ion-channel, p7; and at least six nonstructural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (for a review, see reference 23). Standard-of-care therapy with pegylated alpha interferon and ribavirin has only limited efficacy in eliminating the infection and is relatively toxic. There is thus an important need for new and potentially better therapeutic interventions (33). A greater understanding of the host factors involved in HCV replication may facilitate development of novel, host-specific therapies that are less likely to engender drug resistance. One such host factor is miR-122, a well-conserved, highly abundant, liver-specific microRNA (miRNA) that facilitates HCV genome amplification by an uncertain mechanism that involves a direct interaction with positive-sense viral RNA (14, 15). Indeed, recent studies have demonstrated a remarkable antiviral effect in chimpanzees following therapeutic silencing of miR-122 by administration of a locked nucleic acid (LNA) antisense oligonucleotide (21).

miRNAs are approximately 22-nucleotide-long, single-stranded RNAs of endogenous origin that posttranscriptionally regulate gene expression, typically by mediating mRNA degradation and/or translational blockade after binding to complementary sequences in the 3′ nontranslated region (3′UTR) of the target mRNA (6). miR-122 is expressed at high abundance in adult human liver and also in Huh-7 cells, a human hepatoma cell line that is commonly used for propagation of HCV (2). miR-122 downregulates the expression of cationic amino acid transporter (CAT-1) (2). It also modulates the expression of genes involved in hepatic lipid and cholesterol metabolism (4, 20), processes intricately linked to HCV replication and infectious virus production (9, 37).

How miR-122 promotes the replication of HCV RNA is not understood. It binds to conserved sites within the 5′UTR of the HCV genome, which contains overlapping *cis*-acting signals involved in translation and RNA synthesis (reviewed in reference 23). Phylogenetic, biochemical, and mutational analyses suggest that the 5′UTR is comprised of four highly conserved structural domains (11, 25). The extreme 5′ 125 nucleotides, encompassing domains I and II, contain signals that are essential for RNA synthesis, with the remainder of the 5′UTR contributing in a less essential manner to the overall efficiency of RNA replication (8). These downstream 5′UTR sequences also contain an internal ribosomal entry site (IRES) that allows for cap-independent assembly of the 48S ribosomal complex on viral RNA with only a minimal requirement for canonical translation factors (24, 30). miR-122 interacts with two well-conserved, tandem binding sites, each complementary to the "seed" sequence (nucleotides [nt] 2 to 7) of miR-122 and located in close proximity to each other between stem-loops I and II of the 5′UTR (Fig. 1A) (14, 15). Mutational studies

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▽ Published ahead of print on 28 April 2010.

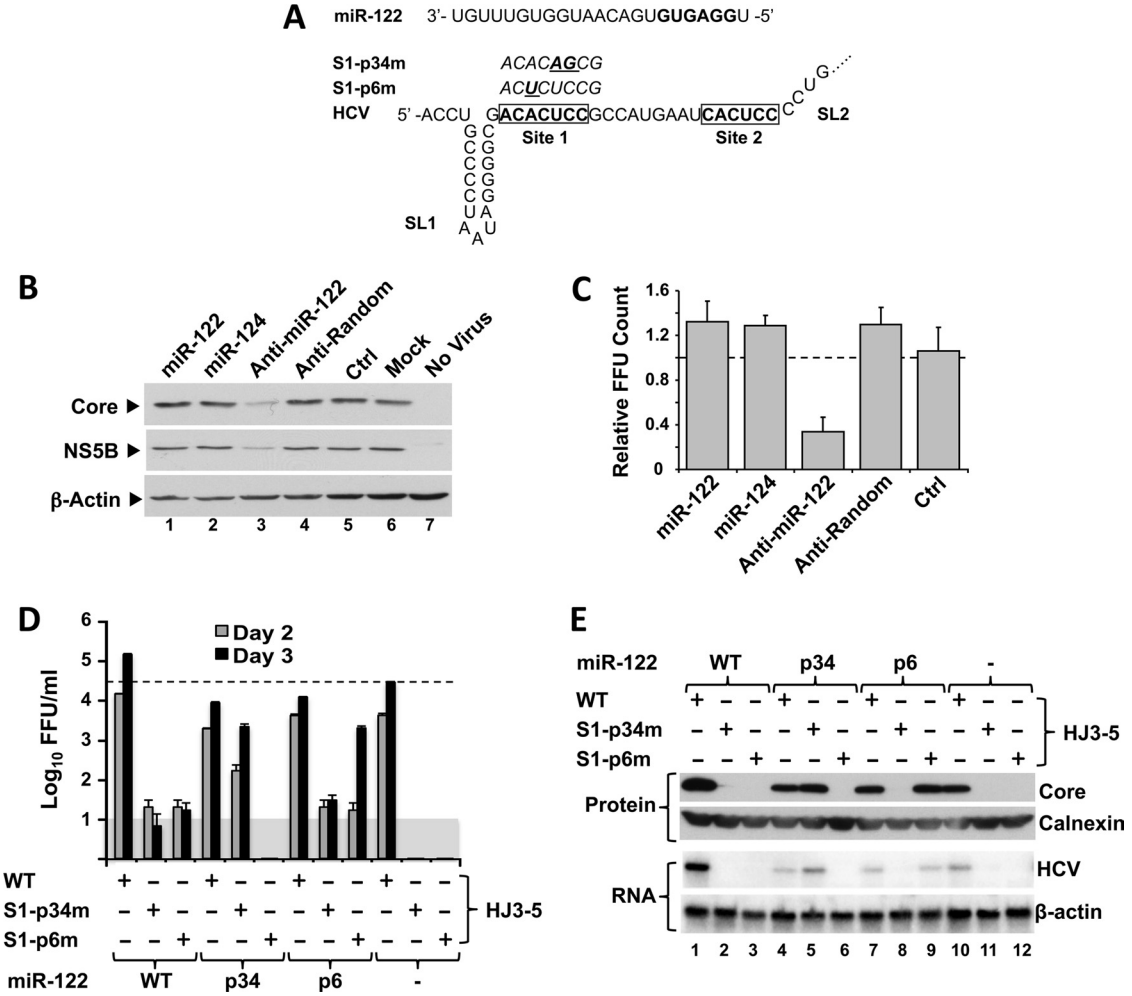

FIG. 1. The replication of infectious HCV is dependent on direct interaction of miR-122 with the RNA genome. (A) Schematic representation of the two miR-122-binding sites, S1 and S2, located between stem-loop (SL) I and II in the 5′UTR of the HJ3-5 genome. At the top is shown the sequence of miR-122. The miR-122 seed sequence and miR-122 complementary sequences in HCV RNA (boxed) are highlighted in bold. The sequences of the S1-p34m and S1-p6m mutants are shown in italic, with base substitutions underlined. (B) Huh-7.5 cells were transfected with the indicated RNA oligonucleotides at 50 nM and 24 h later were infected with HJ3-5 virus, an intergenotypic chimeric HCV, at an MOI of 1. At 72 h postinfection, cell lysates were prepared and subjected to immunoblotting using β-actin, HCV core and NS5B-specific antibodies. (C) Huh-7.5 cells in eight-well chamber slides were transfected with the indicated RNA oligonucleotides at 50 nM and 24 h later were infected with 100 to 120 FFU of chimeric HJ3-5 virus. Seventy-two hours later, cells were labeled for HCV core antigen and individual foci of infected cells enumerated by immunofluorescence microscopy. The results shown represent the means from three independent experiments, each performed in duplicate, ± standard deviations (SD). (D) miR-122 regulates infectious virus production by HCV RNA-transfected cells. FT3-7 cells were transfected with wild-type (WT), p34 mutant, or p6 mutant form of miR-122 or were mock treated. Twenty-four hours later, cells were transfected with wild-type or mutant HJ3-5 HCV RNAs, followed by another transfection of miR-122 and related mutants at 48 h. Supernatant fluids collected 2 and 3 days after HCV RNA transfection were assayed for infectious virus by FFU assay on naïve Huh-7.5 cells (average ± SD; $n = 2$). (E) Immunoblots (top panel) and Northern blots (bottom panel) of extracts from the cells in panel D prepared 3 days after HCV RNA transfection.

suggest that both sites are likely to be occupied by miR-122 in replicating viral RNAs (14). However, while both are involved in the promotion of genome amplification by miR-122, the relative contribution of each site to this process is unknown.

Although mutational analyses of the interactions of miR-122 with HCV RNA have shown their importance in viral RNA amplification, such studies have not been done in the context of systems producing fully infectious virus. Thus, although the ability of miR-122 to enhance the abundance of viral RNA in replicon systems is known to be largely if not entirely dependent upon its direct interaction with viral RNA (15), the same cannot be said for how miR-122 promotes the production of infectious virus. Previous studies do not exclude an additional indirect role for miR-122 in this process, as miR-122 could also regulate the expression of cellular genes required for viral entry, viral assembly, or egress of infectious virus.

The mechanism by which the interaction of miR-122 with the HCV genome facilitates an increase in its abundance is also poorly understood and a subject of controversy. While Jopling et al. (14, 15) found no evidence that miR-122 specifically enhances HCV IRES-directed translation, Henke et al. (10) reported that it upregulates the translation of short RNAs

comprising a reporter protein sequence flanked by the viral UTRs. The relevance of these observations to the situation with the full-length viral genome is uncertain, however, as similar experiments with genome-length HCV RNA showed only minimal (<2-fold) upregulation of translation by miR-122 (10). How much this translational effect might contribute to the striking enhancement of genome amplification by miR-122 is unknown. Moreover, the need for direct binding of miR-122 to promote translation of the full-length viral genome and the relative contributions of the two miR-122-binding sites within the HCV 5′UTR to viral translation have yet to be determined.

We set out to study these issues using a laboratory strain of HCV (HJ3-5 virus) that replicates efficiently in cultured hepatoma cells (39) and is also infectious in the chimpanzee (M. Yi and S. M. Lemon, unpublished data). Our results show that the ability of miR-122 to promote the growth of infectious virus is dependent upon its direct interaction with both seed sequence-binding sites in the 5′UTR. However, binding to the 5′ S1 site is more important for efficient replication than binding to the nearby S2 site. We also find that miR-122 positively regulates HCV translation and that this is also dependent upon direct interactions of miR-122 with both the S1 and S2 sites.

MATERIALS AND METHODS

Cell cultures and virus. Huh-7, Huh-7.5, and FT3-7 cells (another clonal derivative of Huh7 cells that efficiently supports HCV replication) were maintained as described previously (39). Experiments with cell culture infectious virus were carried out with vH-NS2/NS3-J/Y361H/Q1251L (referred to here as HJ3-5) virus, an intergenotypic chimeric virus in which sequence encoding core-NS2 of the genotype 1a H77c virus was placed within the background of the genotype 2a JFH-1 virus (27, 39). Huh-7.5 cells (plated at 40% confluence 1 to 2 days previously) were infected with HJ3-5 virus at multiplicities of infection (MOI) ranging from 1 to 2. Infectious virus yields were determined by a fluorescent-focus assay, with results reported as focus-forming units (FFU)/ml, as described previously (40). For rescue of virus from *in vitro*-transcribed RNAs, FT3-7 cells seeded into six-well plates were transfected with 1.25 to 2.50 μg HJ3-5 RNA per well using the *Trans*IT mRNA transfection reagent (Mirus Bio) for 6 h as per the manufacturer's protocol. After being washed with phosphate-buffered saline (PBS), the cells were fed with fresh medium, which was replaced at 24-h intervals.

Plasmids and *in vitro* RNA transcription. HCV mutants were generated in the background of pHJ3-5 (27, 39). miR-122-binding site and IRES subdomain mutants were constructed using an overlapping PCR strategy with primers containing the corresponding mutations. For translation studies, pHJ3-5 was modified using PCR-based mutagenesis to create an in-frame insertion of the *Renilla* luciferase (RLuc) sequence, fused to the foot-and-mouth disease virus (FMDV) 2A autoprotease, between the p7 and NS2 sequences of HJ3-5, similar to a luciferase reporter virus described by Jones et al. (13). HCV RNA was transcribed *in vitro* from plasmid DNA using reagents provided with the T7 MEGAscript kit (Applied Biosystems) and purified using the RNeasy mini kit (Qiagen). The RNA products were analyzed by denaturing agarose gel electrophoresis to ensure their quality. A capped synthetic RNA transcript encoding firefly luciferase (FLuc) and containing a 30-nt-long 3′ poly(A) tail was produced by *in vitro* transcription from a PCR product containing a T7 promoter. FLuc RNA was transfected along with HCV RNA as an internal control for transfection and translation.

Antibodies. Immunoblotting was carried out using the following antibodies: mouse monoclonal antibody (MAb) to core protein (C7-50; Affinity BioReagents), rabbit polyclonal anti-NS5B (catalog number 266-A; Virogen), rabbit polyclonal anticalnexin (Sigma), and mouse monoclonal anti-β-actin (AC-74; Sigma). The immunoblots shown in Fig. 1 and 2 were probed by horseradish peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (ECL-Plus kit; Amersham Pharmacia Biotech). IRDye 800CW goat anti-mouse and IRDye 680 goat anti-rabbit secondary antibodies (Li-Cor Biosciences, Lincoln, NE) were used to probe the immunoblots shown in Fig. 5, followed by scanning with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

RNA oligonucleotides. RNA oligonucleotides and 2′-O-methylated oligonucleotides were synthesized by Dharmacon. The sequences for single-stranded RNA (ssRNA) oligonucleotides were as follows: miR-122-ss (wild-type miR-122), 5′-UGG AGU GUG ACA AUG GUG UUU GU-3′; miR-122p34-ss, 5′-UGC UGU GUG ACA AUG GUG UUU GU-3′; miR-122p6-ss, 5′-UGG AGA GUG ACA AUG GUG UUU GU-3′; miR-122*-ss, 5′-AAA CGC CAU UAU CAC ACU AAA UA-3′; miR-124-ss, 5′-UUA AGG CAC GCG GUG AAU GCC A-3′; miR-124*-ss, 5′-CCG UGU UCA CAG CGG ACC UUG A-3′; anti-miR-122 (122-2′-O-Me), 5′-AGA CAC AAA CAC CAU UGU CAC ACU CCA CAG C-3′; and antirandom (Rand-2′-O-Me), 5′-CAC GUU AAA ACC AUA CGC ACU ACG AAA CCC C-3′ (13). Mature miRNA duplexes were generated by annealing equimolar amounts of the ssRNA oligonucleotides as follows: wild-type miR-122, miR-122-ss, and miR-122*-ss; miR-122p34, miR-122p34-ss, and miR-122*; miR-122p6, miR-122p6-ss, and miR-122*-ss; and miR-124, miR-124-ss, and miR-124*-ss. miRNAs were always transfected as duplexes, while the antisense anti-miR-122 and antirandom were transfected as single-stranded oligonucleotides. A nontargeting pool of four siRNAs (ON-TARGET-*plus*; Dharmacon), referred to here as Ctrl, has no known targets within mammalian genomes and was used as an additional negative control.

Northern blotting. Total RNA, isolated using the RNeasy mini kit (Qiagen), was subjected to Northern blotting with HCV- and β-actin-specific ^{32}P-labled RNA riboprobes (NorthernMax kit; Applied Biosystems). Briefly, 3 to 10 μg of total RNA was resolved on denaturing formaldehyde-agarose (0.9%) gels, followed by downward capillary transfer to a BrightStar-Plus nylon membrane (Applied Biosystems). Following UV cross-linking, membranes were hybridized with the riboprobes overnight at 68°C. The membranes were extensively washed and analyzed with a PhosphorImager (Storm 860; Molecular Dynamics). HCV RNA was detected with a riboprobe specific to the entire HCV 5′UTR.

miR-122 supplementation and HCV replication. FT3-7 cells in a six-well culture plate were transfected with miRNA duplexes at 50 nM using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. At 24 h, the cells were retransfected with *in vitro*-transcribed HCV RNA (1.25 to 2.50 μg/well) for 6 h using the *Trans*IT mRNA transfection reagent (Mirus Bio) according to the manufacturer's protocol. The cells were transfected with miRNA once again 24 h later and fed with fresh medium every 24 h, and supernatant fluid samples were collected on days 2 and 3 after HCV RNA transfection for virus titration.

To assess the replication of HCV genomes expressing RLuc, FT3-7 cells transfected with miRNA duplexes the previous day (as described above, in six-well plates) were cotransfected with HJ3-5/RLuc2A HCV RNA (1.25 μg/well) and FLuc mRNA (0.25 μg/well) and supplemented again with miRNAs 24 h later. RLuc and FLuc assays were carried out on cell lysates prepared at various time points after HCV RNA transfection using the dual luciferase assay kit (Promega). RLuc activity was normalized to FLuc activity and results presented as fold increase over the RLuc/Fluc ratio at 8 h.

miR-122 supplementation and HCV translation. Huh-7.5 cells seeded into six-well plates were transfected with RNA oligonucleotides at 50 nM as described above. Twenty hours later, replication-deficient HJ3-5/RLuc2A-GND mutant RNA (1.25 μg/well) was cotransfected with capped and polyadenylated FLuc mRNA (0.25 μg/well) using the *Trans*IT mRNA transfection reagent (Mirus Bio). Cells were harvested for dual luciferase assays, in duplicate, at the indicated time points. Results are expressed as the ratio of RLuc to FLuc activity at each time point.

RESULTS

miR-122 enhances production of infectious virus through direct interactions with positive-sense RNA. Randall et al. (31) reported that silencing miR-122 by transfection of an antisense oligonucleotide reduces the yields of infectious virus. Consistent with this, we observed reduced expression of viral proteins (core and NS5B) in cells transfected with anti-miR-122, a 2′-O-methylated antisense oligonucleotide capable of sequestering and causing functional knockdown of endogenous miR-122, after infection with a chimeric genotype 1a/2a virus, HJ3-5 (Fig. 1B). We also noted a 65% reduction in foci of infected cells when we infected anti-miR-122-transfected cells with HJ3-5 virus at a low multiplicity of infection (Fig. 1C). Importantly, we did not note any enhancement in protein expression

or infectious focus formation when cells were supplemented with additional synthetic miR-122, suggesting that the normal endogenous abundance of miR-122 is not always limiting for the replication of infectious virus in these cells (a modest increase in infectious virus yield was observed following miR-122 supplementation in subsequent experiments, as shown in Fig. 1D and described below). We noted no differences in HJ3-5 replication in cells transfected with an unrelated miRNA, miR-124; a randomized antisense oligonucleotide, antirandom; or Ctrl siRNA (a pool of four nontargeting siRNAs with no known targets in mammalian genomes). Since miR-122 could potentially influence the expression of host cell genes required for virus entry, assembly, or release, we studied the impact of miR-122 on the production of infectious virus by mutated HJ3-5 RNAs containing point mutations in the most 5′ miR-122-binding site: S1-p34m and S1-p6m (Fig. 1A). RNA was transcribed *in vitro* from these constructs and transfected into cells in parallel with wild-type HJ3-5 RNA and a replication-defective mutant with a GND substitution in the NS5B polymerase. Like for the GND mutant (data not shown), no infectious virus could be detected in supernatant fluids from cells transfected with the S1-p34m or S1-p6m RNA, while HJ3-5 RNA-transfected cells produced $\sim 10^4$ FFU/ml (Fig. 1D). In addition, no core protein expression was evident at 3 days after transfection of either the S1-p34m or S1-p6m mutant, nor was any viral RNA detected by Northern blotting (Fig. 1E). These data indicate that the integrity of the 5′ S1 miR-122-binding site is critically important for replication of infectious HCV.

To confirm that mutations in the S1 site impair HCV production by ablating the binding of miR-122, we attempted to rescue replication of the mutants by prior transfection of the cells with miR-122 mutants, p34 and p6, having sequences complementary to the modified binding site sequences in the S1-p34m and S1-p6m viral genomes, respectively. In both cases, transfection of the related miR-122 mutant specifically enhanced infectious virus yields over 100-fold by 3 days after transfection of the HCV RNA (Fig. 1D). Since the virus titrations were carried out in naïve Huh-7.5 cells that were not supplemented with the mutated miR-122s, the results shown in Fig. 1D are likely to underestimate the absolute level of production of infectious virus by the mutant RNAs (see below). Consistent with this, the size of foci of infected cells was significantly reduced for the mutant viruses, and infected cells also stained less brightly due to a lesser abundance of core protein compared with cells infected by the wild-type virus (data not shown). Transfection of the mutant miR-122s also rescued core protein expression by the mutant RNAs and increased viral RNA abundance (Fig. 1E).

In these experiments, prior supplementation of cells with additional wild-type miR-122 increased the yield of infectious wild-type virus by about 4-fold and also resulted in the production of minimal but detectable quantities of infectious p6m and p34m virus (Fig. 1D). Similarly, supplementation with the miR-122p6 mutant resulted in the production of small amounts of infectious S1-p34m virus. Importantly, however, viral RNA, protein expression, and infectious virus yields from the mutated genomes were restored to levels approaching those of the wild-type virus after supplementation with the corresponding mutated forms of miR-122 (Fig. 1D and E). These results indicate that the production of infectious virus is dependent on direct interactions of miR-122 with the HCV genome, most likely due to the positive influence of miR-122 on viral genome amplification (15).

Relative importance of the two 5′UTR miR-122-binding sites in HCV replication. Jopling et al. (14) recently reported that both miR-122-binding sites (S1 and S2) are occupied by miR-122 in replicating viral RNAs, and they suggested that both sites are equally important for viral RNA replication. To assess the importance of the S2 site in the viral life cycle, we created a series of HJ3-5 virus mutants with single-base substitutions (p6m) in either or both miR-122-binding sites (Fig. 2A) and determined their ability to replicate following transfection as RNA into cells supplemented with wild-type miR-122, the complementary miR-122p6 mutant, or an unrelated miRNA, miR-124. Replication of the S1-p6m mutant and the S1-S2-p6m double mutant was severely impaired in either miR-122- or miR-124-supplemented cells, as no viral RNA or core protein could be detected by 3 days after transfection of the viral RNA (Fig. 2B). However, we did detect a small amount of infectious S1-p6m virus when we inoculated supernatant fluids from miR-122-supplemented cultures onto naïve Huh-7.5 cells (Fig. 2C). This confirms the earlier results shown in Fig. 1D and indicates that the integrity of the conserved S1 sequence is not absolutely essential for virus replication. To more accurately quantify the infectious virus produced from the mutant genomes, we supplemented Huh-7.5 cells used in the infectious virus titration (FFU) assay with the mutant miR-122p6. This resulted in nearly a 10-fold increase in the titer of the infectious S1-p6 virus detected in harvests from the miR-122-supplemented cells (to just over 10^2 FFU/ml) and also revealed a small quantity of infectious virus produced in cells supplemented with the control miR-124 (Fig. 2C). However, even under these conditions, we were unable to detect infectious virus produced by the double mutant, S1-S2-p6m.

In sharp contrast, viral RNA containing a similar one-base substitution in the downstream miR-122 binding site (S2-p6m) replicated surprisingly well, producing detectable core protein and viral RNA (Fig. 2B) and infectious virus yields almost 1,000-fold greater than those of the S1-p6m mutant in cells supplemented with wild-type miR-122 (Fig. 2C). Unlike the S1-p6m mutant, infectious S2-p6m virus was readily detected in supernatant fluids from cells supplemented with the control miR-124, even when the indicator cells used in the infectivity titration were not supplemented with the mutant miRNA, miR-122p6. These results demonstrate that the downstream S2 site, while important for viral replication, is significantly less essential than the upstream S1 miR-122-binding site. With the double mutant, S1-S2-p6m, core protein expression, viral RNA (Fig. 2B), and production of infectious virus (Fig. 2C) could be demonstrated only when the RNA was transfected into cells supplemented with the mutant miR-122-p6 (and infectious virus yields determined in infectivity assays using similarly supplemented indicator cells). Taken in aggregate, these results indicate that at least one binding site for miR-122 (or a mutated form of miR-122 if expressed in the cell) must exist for virus replication to occur and that to some extent the two sites function redundantly albeit at lower efficiency when one or the other is mutated. A loss of the S2 site is readily compensated for by supplementation of cells with additional miR-122, while

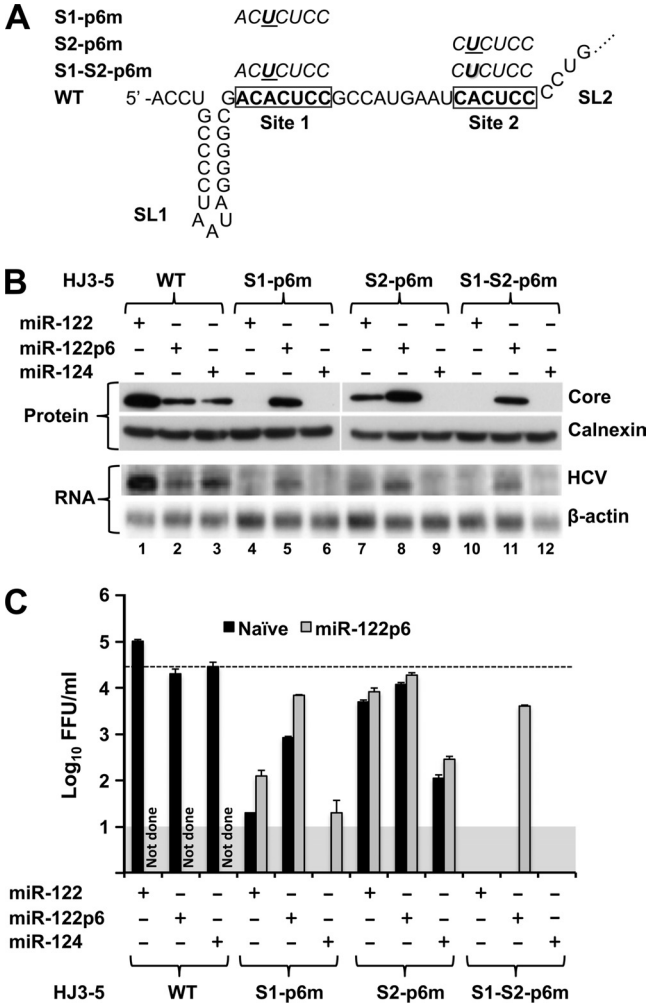

FIG. 2. Relative importance of the two 5′UTR miR-122-binding sites for production of infectious virus. (A) Schematic representation of the S1 and S2 miR-122-binding site mutant sequences. (B) FT3-7 cells were transfected with the indicated miRNA, retransfected with HJ3-5 RNAs 24 h later, and transfected once more with miRNAs 24 h after that. Samples prepared 3 days after HCV RNA transfection were subjected to immunoblot analysis of HCV core protein, with calnexin run in parallel as a loading control (top panel), and Northern blotting for HCV RNA using β-actin mRNA as a loading control (bottom panel). (C) Infectious virus yields 3 days after HCV RNA transfection of the cells in panel B, as quantified by a fluorescent infectious focus assay using naïve or miR-122p6-supplemented Huh-7.5 cells transfected with miR-122p6 at 50 nM 24 h prior to sample inoculation. Data shown are from a representative experiment.

this is not the case with the most 5′ S1 site, on which the replication of the virus is more dependent.

Binding of miR-122 to the S1 site promotes HCV translation. Significant controversy exists concerning the potential effect of miR-122 on HCV translation. While Jopling et al. (14, 15) could not demonstrate any increase in HCV IRES-directed translation due to miR-122, Henke et al. (10) reported striking upregulation of the translational activity of short reporter RNAs by miR-122 but noted only small increases in translation of viral genomes and did not link the latter to a requirement for miR-122 binding. To better characterize the impact of miR-122 on translation of the viral genome, we introduced sequence encoding *Renilla* luciferase (RLuc) (fused at its 3′ end to the FMDV 2A autoprotease) between the p7 and NS2 sequences of HJ3-5 virus (Fig. 3A). To knock out the capacity of this RNA to replicate, we engineered a GND mutation within the active-site GDD motif of the NS5B polymerase to create HJ3-5/RLuc2A-GND (referred to below as "GND" for simplicity). We transfected this modified virus RNA into cells along with a capped and polyadenylated control mRNA encoding firefly luciferase (FLuc) and determined the ratio of RLuc activity to FLuc activity expressed at timed intervals as a measure of IRES-directed translation. As shown in Fig. 3B (left panel), IRES activity peaked at between 6 and 8 h after transfection in the absence of prior supplementation of the cells with miR-122 and was increased by 80 to 90% if the cells were supplemented with miR-122 prior to transfection of the GND RNA. Conversely, sequestration of miR-122 by prior transfection of an antisense oligonucleotide reduced HCV IRES-directed translation by 60 to 80%. However, prior transfection of the cells with the mutant miR-122p6 or an antisense oligonucleotide targeting random sequence had no effect on the efficiency of HCV translation (Fig. 3B, left panel). These data confirm the results of Henke et al. (10) and suggest that miR-122 does indeed have a moderate positive effect on HCV translation.

To determine whether the promotion of HCV translation by miR-122 requires a direct interaction with the viral genome, we introduced an S1-p6m mutation (single-base substitution) (Fig. 2A) into the GND construct shown in Fig. 3A and repeated these experiments as described above. In the absence of prior miR-122 supplementation, this GND-S1-6 pm mutant RNA demonstrated reduced translational activity compared with GND RNA (Fig. 3B, compare right and left panels). Peak expression of RLuc from the GND-S1-p6m mutant also occurred somewhat earlier than that from the related GND RNA, raising the possibility that the transfected mutant RNA might be less stable in the cells. Remarkably, however, the translational activity of the GND-S1-p6M mutant was significantly enhanced by prior supplementation with the related mutant miRNA miR-122p6 and was only minimally influenced by supplementation with wild-type miR-122. Unlike for the GND RNA containing a wild-type S1 site (Fig. 3B, left panel), sequestration of wild-type miR-122 also had only a minimal effect on translation of the GND-S1-p6m mutant. These results indicate that the ability of miR-122 to upregulate the translation of HCV RNA is dependent on its interaction with the S1 miR-122-binding site within the 5′UTR. Interestingly, peak RLuc expression from the GND-S1-p6m mutant was delayed in cells supplemented with miR-122p6 (7 to 8 h, versus 5 to 6 h in nonsupplemented cells) (Fig. 3B, right panel), suggesting possible stabilization of the transfected RNA. We investigated this directly by monitoring the abundance of the transfected GND RNA by an RNase protection assay, but we did not observe any differences related to miR-122 supplementation or sequestration (data not shown).

Contribution of the S1 versus the S2 binding site to miR-122 promotion of viral translation. To determine the relative importance of the two seed sequence-binding sites in the 5′UTR for miR-122 regulation of HCV translation, we created additional replication-deficient GND mutants with p6m mutations

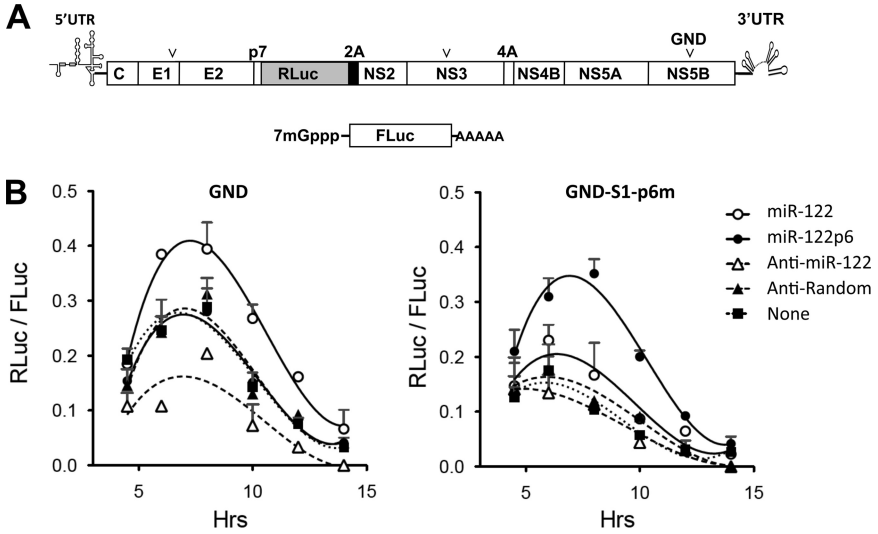

FIG. 3. Binding of miR-122 to the S1 site promotes HCV translation. (A) Schematic representation of HJ3-5/RLuc2A-GND (referred to as GND in the text), which contains an in-frame insertion of the RLuc luciferase sequence fused to FMDV 2A (RLuc2A) between p7- and NS2-coding regions of HJ3-5 and a replication-lethal Asn substitution within the active site of the NS5B RNA-dependent RNA polymerase. (B) Duplicate cultures of Huh-7.5 cells in a six-well plate were transfected with miR-122 to supplement endogenous miR-122 levels or with a 2′-O-methyl antisense RNA, anti-miR-122, to functionally sequester endogenous miR-122. Twenty hours later, GND (left panel) or GND-S1-p6m (right panel) HCV RNA (1.25 μg/well) was transfected together with a capped and polyadenylated FLuc mRNA (0.25 μg/well). Dual luciferase assays were carried out on lysates prepared at the intervals noted following HCV RNA transfection, with results expressed as the mean RLuc/FLuc ratio (± SD) at each time point. Solid and dashed lines are best-fit third-order polynomial plots (r^2 of between 0.78 and 0.99). The data shown are representative of multiple experiments.

in either the S2 or both the S1 and S2 binding sites (Fig. 2A). We then transfected these along with the GND RNA (containing wild-type S1 and S2 sites) and the GND-S1-p6m mutant into cells supplemented with wild-type miR-122, miR-122p6, or miR-124. The translation of all three mutants was substantially reduced compared to that of GND RNA in miR-124-supplemented cells and was increased (but not fully restored to the level of the parental GND construct) in cells supplemented with the miR-122p6 mutant (Fig. 4A). In contrast to the marked differences in virus yield between HJ3-5 viruses containing mutations in S1 and S2 (Fig. 2C), the differences observed in the translational activities of GND-S1-p6m and GND-S2-p6m were minimal. The GND-S1-p6m mutant was only slightly less active than GND-S2-p6m in cells supplemented with wild-type miR-122, while the reverse was apparent in cells supplemented with miR-124. The double mutant, GND-S1-S2-p6m, was only slightly more impaired in translation than either of the single mutants (Fig. 4A). These experiments were done using the *Trans*IT mRNA transfection reagent (Mirus Bio), but similar results were obtained with electroporation of the RNAs (data not shown). We conclude that the two miR-122 seed sequence-binding sites function with roughly equal importance in miR-122 regulation of HCV translation.

We also assessed the impact of the S1 and S2 mutations on the replication capacity of HJ3-5/RLuc2A constructs lacking the GND mutation. While the insertion of the reporter protein sequence in HJ3-5/RLuc2A impaired replication of the viral RNA overall, exponential increases in RLuc activity were nonetheless evident by 76 h after RNA transfection and were significantly stimulated by supplementation of the cells with miR-122 (Fig. 4B, left panel). No such late increase in RLuc expression was observed following transfection of the cells with HJ3-5/RLuc2a-S1-p6m and HJ3-5/RLuc2a-S1-S2-p6m unless the cells were first supplemented with the mutant miR-122p6 (Fig. 4B). In contrast, the S2-p6m mutant remained partially responsive to the wild-type miR-122, consistent with the dominant role of the S1 binding site in miR-122 regulation of HCV replication (Fig. 3).

Is the influence of miR-122 on IRES-directed translation sufficient to account for its regulation of HCV replication? Although the data shown in Fig. 4 demonstrate that miR-122 promotes translation directed by the HCV IRES and that this is dependent upon its direct interaction with viral RNA, they leave unanswered the question of whether miR-122 positively regulates any other step in the viral life cycle. We asked if the 65 to 75% reduction in translation that we observed with the GND-S1-p6m and GND-S1-S2-p6m mutants (Fig. 4A) was sufficient to account for the 100- to 1,000-fold decrease in yields of infectious virus when identical mutations were made in infectious RNAs (Fig. 2C). To answer this question, we generated a series of HJ3-5/RLuc2A constructs containing mutations in the IRES with a range of defects in translational activity. Domain III forms the core of the IRES and interacts with the 40S ribosomal subunit as well as eukaryotic initiation factor 3 (eIF3) during internal entry of the ribosome on the RNA (24). Point mutations in the hairpin loop (-U_{264}UGGGU_{269}-) of stem-loop IIId impair 40S subunit binding and reduce IRES activity to various degrees (16, 18, 19). Since these mutations are located at a substantial distance downstream of *cis*-acting signals in the 5′UTR required for RNA replication (8), we reasoned that any effect they have on infectious virus yield would likely be due to reductions in translation. We constructed six mutants with nucleotide sub-

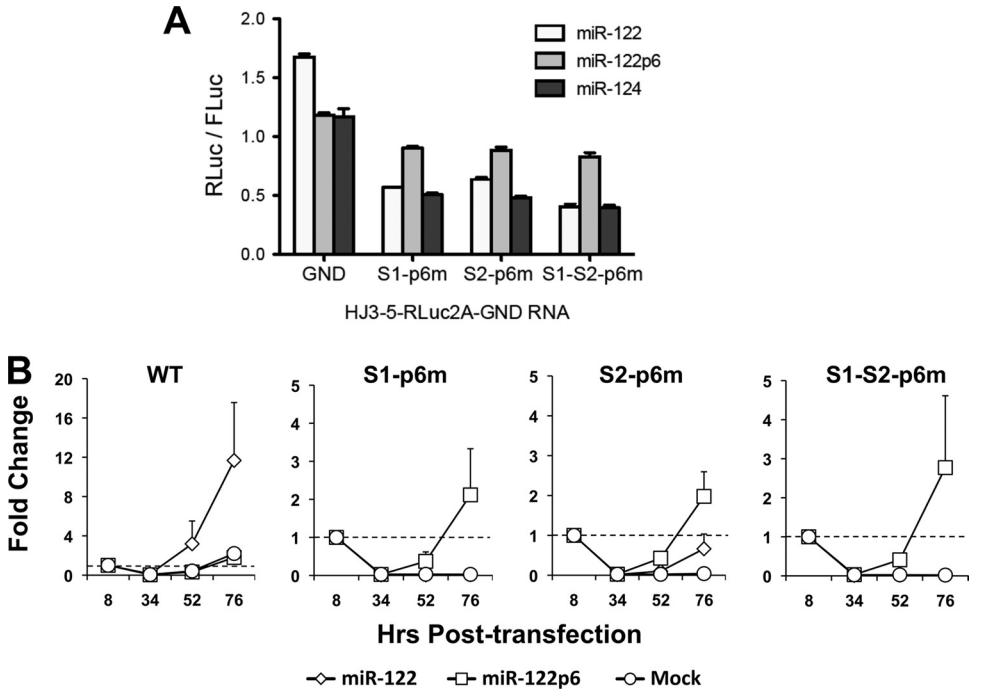

FIG. 4. Contribution of S1 versus S2 binding sites to miR-122 promotion of viral translation versus RNA replication. (A) Huh-7.5 cells were transfected with miR-122, miR-122p6, or miR-124 and cotransfected 20 h later with the indicated GND (see Fig. 3A) and capped control FLuc RNAs. The results shown represent dual luciferase reporter assays of cell lysates prepared 8 h after HCV RNA transfection, presented as the ratio of RLuc to FLuc activity (mean ± SD). Similar results were obtained with 6-h lysates. The data shown are representative of multiple experiments. (B) FT3-7 cells were transfected with miR-122 or miR-122p6 or mock treated and then were retransfected 24 h later with HJ3-5/RLuc2A or related -S1-p6m, -S2-p6m, or -S1-S2-p6m mutant RNAs together with the capped control FLuc RNA. The cells were retransfected with the miRNAs 24 h later. Dual luciferase assays were carried out on lysates prepared 8, 34, 52, and 76 h after transfection of the HCV RNA. RLuc results were normalized to the 8-h FLuc value and are shown as fold increase over RLuc activity at 8 h (mean ± SD; $n = 3$).

stitutions at highly conserved positions within the IIId loop sequence in HJ3-5/RLuc2A-GND (Fig. 5A) and assessed the translational activity of *in vitro*-transcribed RNAs in comparison with the wild type and the miR-122-binding site double mutant, GND-S1-S2-p6m, in transfected Huh-7.5 cells. As reported previously (16, 18), the GND-G266C, -G267C, and -G268C mutants (numbered according to the H77 sequence [GenBank accession no. NC_004102]) were severely impaired for translation, producing RLuc/Fluc activities that were less than 5% of that of the GND RNA (Fig. 5B). On the other hand, translation was only moderately affected in GND-U264A (75 to 80% of wild-type), -U265A (55 to 60%), and -U269A (70 to 75%) mutants. Importantly, the translational activities of GND-U265A and the double binding site mutant, GND-S1-S2-p6m, were comparable (approximately 55 to 60% of that of the wild-type RNA in this series of experiments).

Next, we compared the effect of these IRES mutations with that of the S1-S2-p6m mutation on replication when placed in the background of the HJ3-5 virus genome. When transfected into FT7-3 cells, wild-type HJ3-5 RNA replicated well as evidenced by core protein expression and virus yields of 4×10^4 FFU/ml by 72 h after transfection (Fig. 5C and D). In contrast, the three IRES mutants with severe defects in translation (HJ3-5-G266C, -G267C, and -G268C) produced neither detectable core protein (Fig. 5C) nor infectious virus (Fig. 5D). HJ3-5 RNA containing the U264A and U269A mutations, which caused only a 25 to 30% decrease in translation activity (Fig. 5B), produced minimally less core protein (Fig. 5C) and only 4- to 6-fold less infectious virus than the wild-type HJ3-5 RNA (Fig. 5D). Notably, the U265A mutation, which caused a reduction in translation comparable to that of the S1-S2-p6m mutation, resulted in a barely detectable abundance of core protein in transfected cells and in a reduction in infectious virus yields to approximately 28-fold less than wild-type RNA. These data thus indicate that a 40 to 45% decrease in translation is amplified into a much larger difference in infectious virus yield over the multiple rounds of replication occurring during the 72-h period between RNA transfection and virus harvest in these experiments. Nonetheless, the S1-S2-p6m mutant demonstrated at least a 100-fold-greater replication defect than U265A, generating neither detectable core protein nor any infectious virus. This was reproducible in multiple experiments, even when the yield of infectious virus was determined in cells supplemented with the miR-122p6 mutant (Fig. 2C).

We conclude from these experiments that the 2- to 3-fold decrement in IRES-directed translation resulting from loss of miR-122 binding in the S1-S2-p6m mutant (Fig. 3) contributes significantly to the severe defect in replication of this mutant. However, since the U265A mutant demonstrates a comparable defect in translation yet is still capable of producing infectious virus (up to 1,500 FFU/ml in the experiment shown in Fig. 5D), miR-122 is likely to be essential for some other aspect of HCV genome amplification, either new RNA synthesis or the stability of viral RNA.

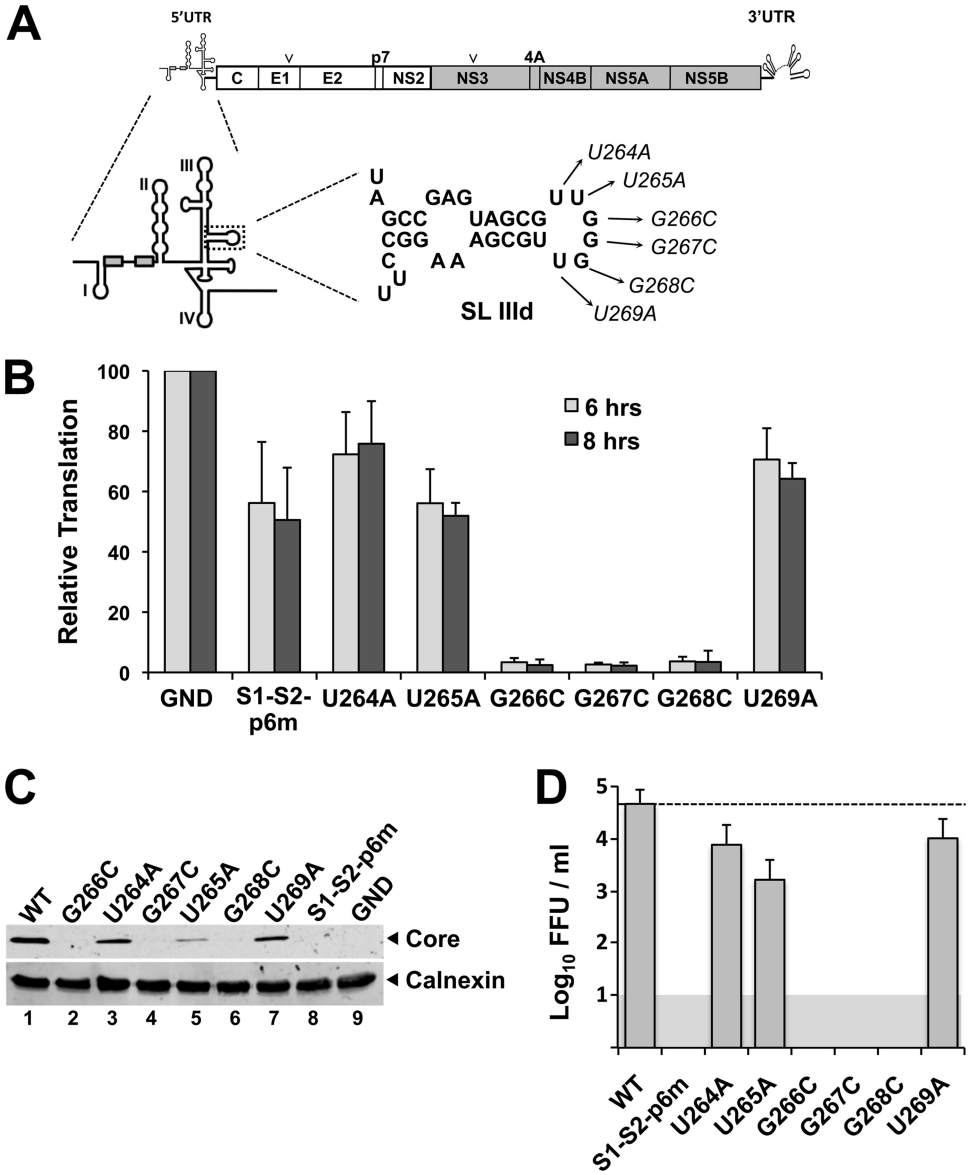

FIG. 5. Comparison of infectious virus yields from the double binding site mutant and IRES mutants with quantitatively similar and different defects in translation. (A) Schematic representation of IRES mutations constructed in stem-loop (SL) IIId of the HJ3-5 5′UTR. The sequence of stem-loop IIId is depicted, with nucleotide positions numbered according to the prototype genotype 1a H77 HCV sequence (GenBank accession no. NC_004102). (B) Translational activities of IRES mutants constructed in HJ3-5/RLuc2A-GND. Cells were cotransfected with GND or the indicated mutant GND RNAs with the capped, polyadenylated FLuc mRNA as an internal control for transfection and cellular translation efficiency. Dual luciferase reporter assays were carried out at 6 or 8 h following transfection, with results expressed as the RLuc/FLuc ratio. The data shown represent the means ± SD from five independent experiments. (C) Immunoblot assays for core protein expressed in FT3-7 cells 3 days after transfection with wild-type (WT) HJ3-5 RNA or related S1-S2-p6m (double miR-122-binding site) and IRES mutants in the HJ3-5 background. (D) Infectious virus yield in supernatant fluids from the HJ3-5 RNA-transfected cell cultures described for panel C. Infectivity titrations were done on fluids collected at 3 days posttransfection and are shown as mean ± range ($n = 2$).

DISCUSSION

miR-122 is an evolutionarily conserved miRNA that is expressed at high abundance in adult human hepatocytes and in Huh-7 human hepatoma cells that are permissive for HCV replication. It is a key regulator of lipid and cholesterol biosynthesis in the liver (4, 5, 20). Interestingly, the HCV life cycle is also intimately linked to lipid and cholesterol metabolism, as products of the cholesterol biosynthetic pathway are essential host factors for HCV replication (36, 38). Cholesterol and lipoproteins are also integral components of virion structure and may play key roles in viral entry (1, 17, 28), while the apolipoprotein secretory pathway is important for egress of virus from infected cells (9). Despite these multiple associations, recent studies indicate that it is possible to uncouple the effects of miR-122 on cholesterol biosynthesis and replication of HCV RNA (29).

The genomic RNA of HCV contains three highly conserved potential miR-122-binding sites that are complementary to the miR-122 seed sequence. These include the 5′-proximal S1 site, located about 22 nt from the 5′ end of the genomic RNA, and the nearby S2 site, located only 16 nt downstream (Fig. 2A). miR-122 facilitates an increase in the abundance of HCV RNA by directly interacting with these tandem S1 and S2 binding sites (14, 15). Both of these sites in the 5′UTR are occupied by miR-122 in replicating RNAs (14). A third potential seed sequence-binding site exists within the 3′UTR, but it has yet to be linked functionally to miR-122. While Jopling et al. (15) first demonstrated that miR-122 was essential for amplification of HCV RNA in cell culture, Randall et al. (31) confirmed that miR-122 is also required for efficient viral replication, as evidenced by a nearly 2-fold reduction in viral RNA levels and infectious virus production following transfection of an miR-122-specific antisense 2′-O-methyl oligonucleotide. Our initial experiments confirmed this finding, demonstrating a 65% decrease in infectious focus formation in similarly treated cells (Fig. 1C). The therapeutic potential of silencing miR-122 has also been demonstrated recently in HCV-infected chimpanzees (21). However, the mechanisms underlying the dependence of HCV replication on miR-122 remain incompletely understood.

To determine whether direct interactions between miR-122 and the HCV 5′UTR are responsible for its positive effect on infectious virus production, we carried out a mutational analysis of the S1 and S2 binding sites in an infectious molecular clone. Mutations in the S1 or S2 binding site, either singly or together, severely hampered the rescue of virus from this clone, reducing infectious virus yields by as much as 10^4 and suggesting that replication of infectious virus is highly dependent on direct interactions of the viral RNA with miR-122 (Fig. 1D and 2C). This was confirmed by demonstrating that the production of infectious virus by these mutants could be restored almost to the level of the parental virus by supplementation of cells with the complementary mutant forms of miR-122 (Fig. 1D and 2C). Thus, most, if not all, of the effects of miR-122 on infectious virus production appear to be mediated via direct interactions of miR-122 with the S1 and S2 sites in the viral 5′UTR. While these data are not surprising, they fill an important gap in our understanding of the mechanism by which miR-122 facilitates growth of the virus.

Recent work by Jopling et al. (14) suggests that both miR-122-binding sites in the 5′UTR are important for the ability of miR-122 to upregulate RNA abundance. Our data, derived from an infectious virus system, confirm roles for both sites but indicate that the most 5′ miR-122 binding site plays a dominant role in replication. Viral RNA with a nucleotide substitution in the S2 binding site (HJ3-5-S2-p6m) produced 10-fold more infectious virus than a mutant with a similarly altered S1 binding site (HJ3-5-S1-p6m) (Fig. 2C). Moreover, supplementation of cells with excess miR-122 drove the production of infectious virus by the S2 mutant almost to the level observed with the parental virus and to 100-fold more than that observed with the S1 mutant under similar conditions. It is difficult to predict whether there are differences in the affinities of the two sites for miR-122, but it seems unlikely that the S2 site would have greater affinity than S1 since, the miR-122-complementary sequence at S2 is one nucleotide shorter (6 nt) than that at S1 (7 nt) (Fig. 2A). Our data thus suggest that the S1 site plays a dominant role in HCV replication and that high miR-122 occupancy at S1 may be capable of driving RNA replication and virus production despite loss of miR-122 binding at S2. The absence of any virus production from the double S1-S2-p6m mutant, even in cells supplemented with excess wild-type miR-122 (Fig. 2C), argues against the possibility that miR-122 might be able to exert a functional effect by binding with low affinity to either the mutated S1 or S2 site.

Substantial controversy concerning the impact of miR-122 on translation directed by the HCV IRES exists. Jopling et al. (15) found that protein expression from monocistronic, full-length viral RNAs containing a mutation in the S1 site that ablated the miR-122 effect on RNA abundance was not measurably reduced from that produced by wild-type RNA in transfected cells. Other studies using various reporter RNAs also failed to demonstrate that miR-122 enhances HCV IRES-directed translation or the stability of HCV RNA (14, 15). Nonetheless, Henke et al. (10) reported that miR-122 is capable of modulating the translation of short reporter RNAs containing both viral UTRs by as much as 3- to 4-fold from the norm. Nonetheless, miR-122 supplementation or sequestration was able to modulate the translation of a full-length, replication-defective RNA only by about 50% (10). Moreover, Henke and colleagues did not determine whether this minor change in translational activity was dependent upon direct interactions of miR-122 with the full-length viral genome. To resolve this controversy, we studied the role of miR-122 binding in translation of a monocistronic, full-length infectious RNA in which sequence encoding a reporter protein (RLuc) had been introduced into the polyprotein-coding region. We ablated the ability of this RNA to replicate by introducing a lethal mutation into the NS5B polymerase and, as an internal control for transfection and cellular translation, cotransfected cells with a capped and polyadenylated mRNA expressing a second reporter (FLuc). Our results demonstrate that miR-122 does indeed positively regulate HCV translation by up to 2-fold and that this is dependent upon the ability of miR-122 to bind to both the S1 and S2 sites which lie upstream of the IRES within the 5′UTR (Fig. 3B and 4A). Importantly, RNase protection assays did not demonstrate changes in the stability of the RNA in these experiments. Translation of a mutated viral RNA with nucleotide substitutions in both the S1 and S2 sites was partially restored by supplementing cells with a cognate miR-122 mutant (Fig. 4A). However, in contrast to the dominant role played by S1 in miR-122 regulation of RNA abundance and infectious virus production, both miR-122 binding sites appeared to be equally important for translation. While Norman and Sarnow (29) reported recently that miR-122 does not modulate the rate of new viral RNA synthesis in transfected cells, the difference in the relative roles of the S1 and S2 sites in translation versus replication that we have documented here indirectly suggests that miR-122 has an HCV-specific function(s) beyond that of promoting viral translation.

Our analysis of an IRES mutant (U265A) with a defect in translation of comparable magnitude to that of the double miR-122-binding site mutant, S1-S2-p6m, indicated that relatively modest reductions in IRES-directed translation are magnified over multiple rounds of replication into large deficits in yields of infectious HCV (Fig. 5). However, it also demon-

strated that the 2- to 3-fold decrease in translational activity observed with the S1-S2-p6m mutant is unlikely to be the only explanation for the inability of this RNA to produce detectable infectious virus. The U265A mutant was impaired only 28-fold in its ability to produce infectious virus, while the defect in virus production by the S1-S2-p6m miR-122-binding site mutant is on the order of 3,000-fold or more (Fig. 4B and 2E). In carrying out this analysis, we assumed that the U265A mutation was unlikely to affect RNA replication, based on its distance downstream of essential replication signals in the 5′UTR (7, 8). However, were this assumption to be incorrect and the U265A mutation were to impair RNA synthesis, it would only strengthen the argument that miR-122 must positively regulate some step in the life cycle of the virus other than translation. There is no basis for suggesting that the U265A mutation might enhance RNA synthesis, which would be an alternative explanation for the discrepancy of the U265A and S1-S2-p6m mutant viruses. These experiments were repeated several times with freshly transcribed RNAs, making it very unlikely that early generation of revertant virus contributed to the replication success of the U265A mutant.

The ability of miR-122 to positively regulate translation was strictly dependent on complementarity between the miR-122 seed sequence and viral RNA (Fig. 4A), indicating that this effect also requires miR-122 binding to the viral genome. Most miRNA-binding sites are located in the 3′UTRs of their target mRNAs and downregulate translation upon recruiting the miRNA-associated silencing complex (miRISC). miRNA-binding sites placed artificially within the 5′UTRs of reporter genes may still repress translation (26). However, the actions of miR-122 on the HCV genome appear to be dependent on the position of the binding site, since the HCV binding site suppresses translation when placed in the 3′UTR of a reporter RNA (10, 14). Therefore, the context in which miR-122 binds to HCV RNA appears to be important to its stimulatory effect on translation. An interesting and yet-to-be answered question is whether miRISC components are required for miR-122 promotion of HCV translation. siRNA-mediated knockdown of miRISC components, including argonaute 1 to 4 (Ago1 to -4) and Rck/p54 (DDX6), impairs the replication of HCV (31, 32), but we have found that Rck/p54 knockdown does not limit the ability of miR-122 supplementation to upregulate HCV replication (12). Thus, it is uncertain whether the miRISC functions in the context of the miR-122 interaction with HCV RNA to recruit viral and/or cellular factors that can promote translation. It is known, however, that under certain conditions, some miRNA interactions with mRNAs may activate rather than repress gene expression. For example, miR-369-3p recruits Ago2 and fragile X mental retardation-related protein 1 (FXR1) to the AU-rich element (ARE) of tumor necrosis factor alpha (TNF-α) mRNA and activates TNF-α translation in serum-starved cells (34, 35).

Alternatively, it is possible that miR-122 binding to the S1 and S2 sites may lead to conformational changes in the HCV IRES that promote translation. A recent study has proposed that miR-122 binding to the 5′UTR increases HCV translation by inhibiting long-range interactions between nt 24 and 38 in the 5′UTR and nt 428 and 442 in the core-coding region (3). While this could change the IRES conformation from a less active "closed" conformation to a more active "open" conformation, miR-122 enhances the amplification of subgenomic replicons that lack the core-coding sequence involved in this long-range intramolecular RNA interaction. This putative conformational switch also cannot explain how miR-122 stimulates translation of reporter RNAs that do not contain the core-coding sequence (10).

In summary, we have shown that the ability of miR-122 to promote the production of infectious HCV is dependent on direct interactions with both of the miR-122-binding sites in the HCV 5′UTR but that the most 5′ S1 site plays a dominant role in this process. We have also shown that miR-122 positively regulates IRES-directed translation of genome-length RNAs by approximately 2-fold and that this is also dependent on its ability to bind to both sites in the 5′UTR. While this provides a partial explanation for the dependence of HCV replication on miR-122, experiments with IRES mutants suggest that the positive regulation of HCV translation by miR-122 is insufficient to fully explain the inability of HCV RNA deficient in miR-122 binding to replicate and produce infectious virus.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (U19-AI40035 and P20-CA150343). R.K.J. was a James W. McLaughlin predoctoral fellow.

We thank David R. McGivern, Tetsuro Shimakami, and Aditya Hindupur for engaging discussions and helpful suggestions.

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